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Differential Role of Long Terminal Repeat Control Elements for the Regulation of Basal and Tat-Mediated Transcription of the Human Immunodeficiency Virus in Stimulated and Unstimulated Primary Human Macrophages

ASHLEE V. MOSES,[1]* CARLOS IBANEZ,[2] RICHARD GAYNOR,[3] PETER GHAZAL,[2] AND JAY A. NELSON[1]

Department of Microbiology and Immunology, Oregon Health Sciences University, Portland, Oregon 97201[1]; Department of Immunology, The Scripps Research Institute, La Jolla, California 92037[2]; and Department of Internal Medicine, South Western Medical School, Dallas, Texas 75235

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Primary human macrophages induced to differentiate through contact with autologous activated nonadherent cells were used to investigate the transcriptional mechanisms involved in reactivation of human immunodeficiency virus (HIV) replication. Through transient transfection experiments with an HIV long terminal repeat (LTR)-chloramphenicol acetyltransferase reporter construct, we show that macrophage differentiation results in a 20-fold upregulation of basal LTR activity. To identify sequence elements responsive to the differentiation process, point mutations introduced into the LTR were tested in differentiated and undifferentiated macrophages. Several elements were identified as positive regulators of basal transcription. TATA, Sp1, and NF-κB binding sites were the most influential. The low-affinity site for LBP-1 (UBP-1) functioned as a negative regulator of LTR activity in undifferentiated macrophages, but this influence was lost upon differentiation. When *tat* was cotransfected into the expression system, the requirement for LTR elements identified as important for positive regulation of basal transcription remained in undifferentiated macrophages. Interestingly, however, the mutations in positive control elements which debilitated activity in undifferentiated macrophages had no effect on LTR activity in differentiated macrophages. Thus, it appears that while HIV-LTR activity is highly dependent on cellular transcription factors in undifferentiated cells, in differentiated macrophages the viral protein Tat confers pliability on the LTR and facilitates autonomy from absolute cellular control mechanisms. In vivo, release from either positive or negative regulation via cellular proteins may facilitate reactivation of HIV in macrophages.

The lifecycle of the human immunodeficiency virus (HIV), the etiologic agent of AIDS, is intimately associated with that of the host cell. Human cells from a variety of tissue systems are susceptible to infection with HIV in vivo, and within these tissues, T cells and cells of the monocyte/macrophage lineage constitute the major targets. HIV has been detected at relatively high frequency in macrophages in the brain (39), lymphatic (1), and lung (3) tissues of AIDS patients. Quiescent macrophages are permissive for proviral synthesis and integration, and even in the absence of cellular activation, the macrophage is able to maintain the virus in a state of postintegration latency (9). Quiescent macrophages may thus constitute an important reservoir of latent virus in HIV-infected individuals, with the potential to reactivate virus for dissemination to distant sites. Indeed, HIV-infected macrophages from asymptomatic individuals can be physiologically induced to reactivate latent provirus when exposed to a T-cell-mediated differentiation stimulus (34). This observation suggests that a macrophage differentiation step is necessary to activate viral replication in vivo. Identification of mechanisms that may transform macrophages from quiescent reservoirs to cells permissive for HIV replication is therefore crucial in elucidating the role of macrophages in AIDS-related disease.

Host transcription factors interacting with specific sequences on the HIV long terminal repeat (LTR) play an integral role in regulating levels of viral gene expression. To date, the majority of studies addressing transcriptional regulation of the HIV LTR have been performed with HeLa cells (2, 7, 8, 13, 15, 20, 40) and T-cell lines (13, 15, 22–26, 28, 30, 31, 33, 40, 41). Additional studies have included a B-cell line (40), a megakaryocyte cell line (33), and the monocytic cell line U937 (15, 24, 28). Studies with primary human cells have been limited to those employing peripheral blood T cells (30, 32). Results from these studies have identified sequence elements on the LTR that are important in regulating the level of viral transcription in either a positive or a negative fashion. Sequences that enhance transcription include, in the U3 region, a tandemly repeated enhancer (−103 to −81) recognized by NF-κB (27) and EBP-1 (40), three Sp1 binding sites (−75 to −47 [8, 19]), the TATA box (−28 to −24) recognized by TFIID and other TATA-binding factors (8), an upregulatory element (URE; −157 to −122 [28]) and in the untranslated leader (R) region, direct repeat sequences recognized by the cellular protein UBP-1 (8) (or LBP-1 [20]), UBP-2 (7), or CTF/NF 1 (20) and the *trans* activation response (TAR) element (+14 to +44), which is responsive to the viral *trans*-activator protein Tat (31). Sequences with the potential to decrease the synthesis of viral RNA include the negative regulatory element (−419 to −157) recognized by cellular

* Corresponding author. Mailing address: Department of Microbiology and Immunology, L220, Oregon Health Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR 97201-3098. Phone: (503) 494-7768. Fax: (503) 494-6862. Electronic mail address: smith@ohsu.edu.

factors including AP-1, NFAT-1, URS, and USF (6, 8, 35) and sequence elements flanking the TATA region which bind UBP-1 (LBP-1) (21). Induction of basal transcription via these cellular factors allows initial expression of the doubly spliced viral mRNAs encoding the regulatory proteins Tat, Rev, and Nef. The Tat protein amplifies LTR-directed gene expression through a specific interaction with the TAR RNA stem-loop structure (5). Cellular factors are also thought to be involved in mediating *tat*-induced gene expression (see reference 10 for a review).

Given the intimate association between viral and host regulatory factors that has been established in other cell systems, in primary macrophages the intracellular environment is likely to play a central role both in the establishment and maintenance of a latent HIV infection and in the reactivation from latency and progression towards lytic (productive) infection. Studies addressing the mechanisms by which HIV is able to establish latency and subsequently reactivate have been limited to monocytic cell lines which do not always accurately reflect the situation in primary macrophages in vivo. To explore the influence of the state of macrophage activation in the progression to productive HIV infection, we have utilized pure cultures of primary human macrophages that are stimulated by contact with mitogen-activated autologous lymphocytes. This study serves to identify specific regulatory elements in the HIV LTR that are operational in activated (stimulated) and quiescent (unstimulated) primary human macrophages and addresses the importance of these elements in the context of viral proteins such as Tat. While these elements have been recognized as functional to differing degrees in other cell systems, the influence of the macrophage activation state on the relative importance of each element suggests a novel adaption of LTR control sequences for the replication strategy of HIV within cells of the macrophage lineage.

MATERIALS AND METHODS

Cells. Peripheral blood mononuclear cells were obtained from the peripheral blood of healthy HIV-seronegative adult donors. Donations were performed at the General Clinic Research Center at the Scripps Research Foundation. Peripheral blood mononuclear cells were isolated, and purified macrophage cultures were prepared as previously described (17). Briefly, cells recovered from a Histopaque (Sigma Chemical Co., St. Louis, Mo.) gradient were plated onto 60-mm^3 Primaria (Becton Dickinson, Lincoln Park, N.J.) culture dishes (5×10^6 to 1×10^7 cells per dish) in 3 ml of Iscove medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 1% penicillin-streptomycin and 10% type AB human serum (Sigma). To generate unstimulated macrophage cultures, adherent cells were allowed to adhere for 90 to 120 min at 37°C in 5% CO_2. To ensure the removal of any residual loosely adherent lymphocytes, cells were rinsed and refed on day 3 or 4 of culture, and any nonadherent cells were discarded. Nonadherent cells were removed by thorough rinsing, and adherent cells were recultured in 60-30 medium (60% AIM V medium [GIBCO]–30% Iscove medium supplemented with 1% penicillin-streptomycin solution and 10% human serum) for 6 days at 37°C in 5% CO_2. To generate stimulated macrophage cultures, adherent cells were incubated overnight in the presence of the nonadherent cells and concanavalin A (Con A; 5 mg/ml; Sigma). Adherent cells were then rinsed and cultured as described for unstimulated cultures. By using this isolation procedure, nonadherent cells were routinely 98% esterase positive at 72 h and expressed the monocyte markers Leu M1, M3, 3a, and 3b. Over the 8-day incubation period, monocytes differentiated into macrophages. Morphological changes consistent with either an unstimulated or a stimulated macrophage phenotype were observed.

Plasmids. Construction of wild-type and mutant HIV LTR chloramphenicol acetyltransferase (CAT) plasmids has been previously described (7, 8, 11, 12, 15, 16, 40). Oligonucleotide-directed mutations introduced into specific LTR elements eliminated the binding of the cognate cellular DNA-binding protein, as shown by transient expression assays and DNase I footprinting. The wild-type LTR and the 11 mutant LTR constructs used are schematically represented in Fig. 1B. Nucleotide numbers are based on the wild-type LTR (-177 to $+78$; WT574), with $+1$ designating the transcription start site. Figure 1 also illustrates target LTR elements and the cellular transcription factors that bind over these regions. Either the wild-type plasmid or one of the mutant plasmids was transfected into macrophages as described below. When noted, cotransfection experiments were performed by using a Tat expression plasmid under the control of the Rous sarcoma virus promoter.

Transient transfections and CAT assays. Macrophages differentiated in culture for 6 days were transfected by a lipofection procedure. Briefly, the test plasmid (5 μg/60-mm^3 culture dish) was mixed with Lipofectin reagent (5 μl per culture dish; Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, Md.) and incubated for 15 min at room temperature. Monolayers were rinsed and incubated in serum-free medium to which the plasmid-Lipofectin mix was added for 4 h. When cotransfection studies were performed, the Tat plasmid (1 μg per culture dish) was separately incubated with Lipofectin (1 μl per culture dish) and added to the monolayer immediately after introduction of the test plasmid. After transfection, cells were gently rinsed and reincubated in 50% fresh medium–50% old medium. Forty-eight hours later, cells were harvested, and lysates were prepared by sonication. Extracts were standardized by counting cell nuclei prior to sonication and by A_{280} readings of lysates. Lysates were assayed for CAT activity by a standard methodology (14). Acetylated and unacetylated forms of [^{14}C]chloramphenicol were separated by thin-layer chromatography and quantitated by liquid scintillation counting in a Beckman LS 5000TD scintillation counter. To compare results from different experiments, CAT activity for each mutant LTR was expressed as a relative percentage of that obtained for the wild-type LTR (adjusted to 100%) for each experiment. Results are presented as the mean ± standard deviation (SD) values of four to six independent experiments using macrophages prepared from different donors.

RESULTS

Basal transcriptional activity in unstimulated and stimulated macrophages. Human monocytes isolated from peripheral blood were stimulated by contact with Con A-activated, nonadherent autologous cells. As previously described (17, 34), stimulated monocytes morphologically differentiated into a variety of cell types, including multinucleated giant cells and cells with ruffled cell borders and pseudopod extensions. Unstimulated macrophages demonstrated a gradual increase in cell volume but retained a spherical, mononucleate appearance (Fig. 2). The differentiation of monocytes into multinucleate giant cells occurred over a 6- to 8-day period. On day 6 of culture, unstimulated and stimulated macrophage cultures were used in 48-h gene transfer experiments with an HIV LTR-CAT reporter construct (WT574). While the HIV LTR was active in unstimulated macrophages (Fig. 3), a 20-fold

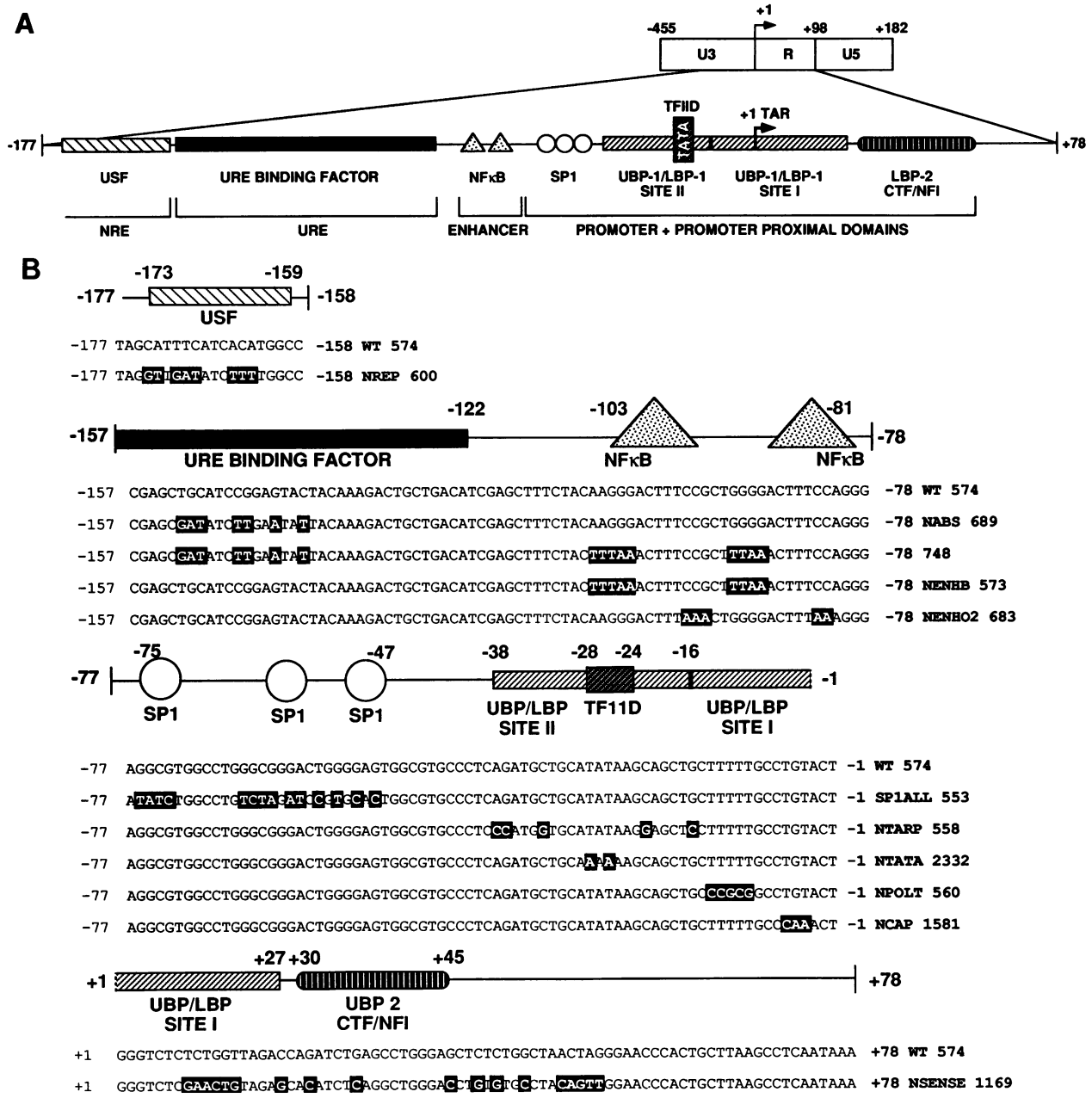

FIG. 1. (A) Schematic map of the U3 and R regions of the HIV LTR relating to the wild-type plasmid construct WT574 (−177 to +78). Nucleotide numbers are relative to the transcription start site at +1. The positions of important *cis*-acting regulatory elements and the cellular DNA-binding factors that recognize these elements are illustrated. (B) Sequential schematic showing the nucleotide sequence of WT574 and the relevant regions of the mutant LTRs aligned with the wild type. Mutated regions are shown in reverse type. The cognate cellular binding factors eliminated by these mutations are shown above.

increase in LTR activity was consistently observed after macrophage stimulation.

The increased transcriptional activity of the HIV LTR in stimulated macrophages may be due to the differential contribution of specific *cis*-acting regulatory sequences on the viral LTR under different conditions of macrophage activation. To identify such potential regulatory elements in the viral LTR, clustered point mutations introduced into specific LTR sequences located between nucleotides −177 and +78 were tested in both stimulated and unstimulated macrophages. The mutations in LTR elements and the cellular factors known to bind these elements are schematically illustrated in Fig. 1. These mutations have been previously characterized and shown to eliminate binding of the cognate cellular factor (7, 8, 11, 15, 16, 40). Figures 4 and 5 graphically represent the transcriptional activity of wild-type and mutant LTR-CAT constructs transfected into unstimulated and stimulated macrophage cultures, respectively. CAT activities for mutant LTRs are expressed as relative percentages of wild-type (WT574) activity.

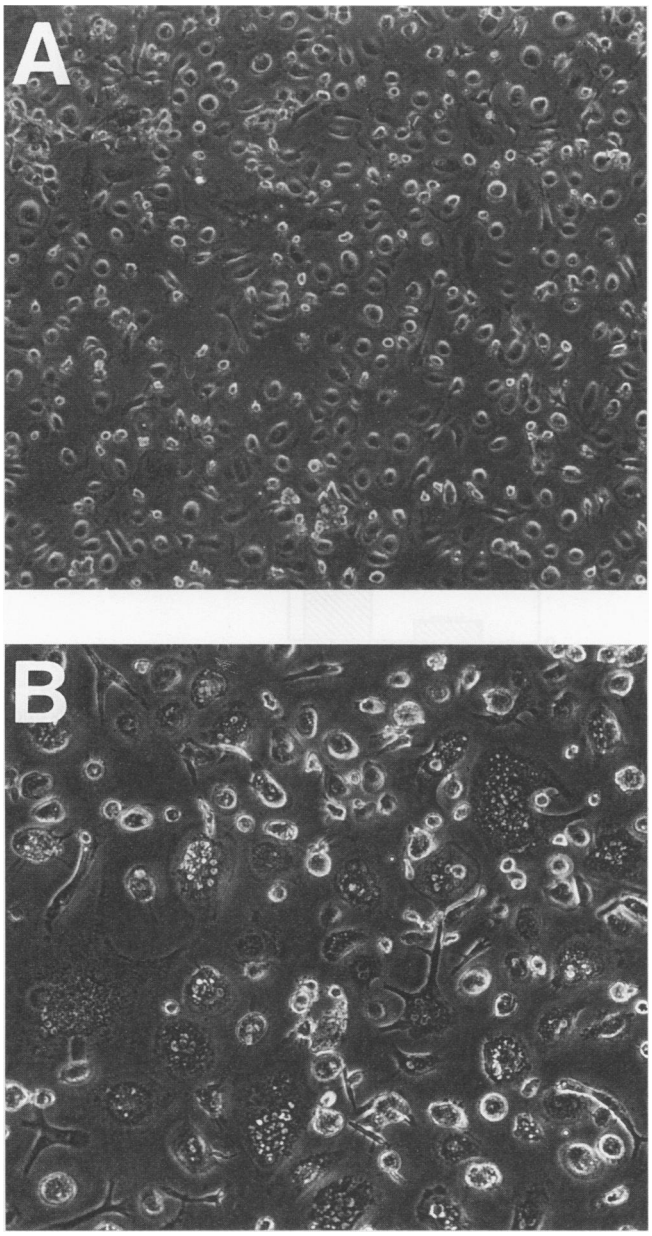

FIG. 2. Morphological appearance of unstimulated (A) and stimulated (B) macrophage cultures at the time (6 days post-culture initiation) of transient transfection. Adherent monocytes were isolated from the peripheral blood of HIV-seronegative donors and cultured for 20 h in the presence (stimulated) or absence (unstimulated) of Con A-activated nonadherent autologous cells, as described in Materials and Methods. Cultured monocytes differentiated into macrophages and were used for transient transfection analysis from day 6 to day 8 of culture.

(i) Analysis of the binding site for the transcription factor USF (nucleotides −177 to −156). The region from −177 to −156 of the HIV LTR contains the sequence that binds the cellular factor USF (−173 to −159). In comparison to the wild-type LTR, mutation of the USF binding site (NREP 600) significantly reduced LTR activity in both unstimulated and stimulated macrophages, indicating that this sequence contributes in a positive manner to maximal transcriptional activity in macrophages regardless of the activation state. It should be noted that the potential to measure a decrease in relative CAT activity is greater in stimulated than in unstimulated macrophages because of the enhanced activity of the wild-type LTR in stimulated macrophage cultures (Fig. 3).

(ii) Analysis of the URE and the enhancer element containing the binding sites for the URE transcription factor and NF-κB (nucleotides −156 to −78). The mutant constructs spanning the region from −156 to −78 include a mutation 5′ to the enhancer in the URE (NABS 689), mutations in the enhancer that destroy NF-κB binding (NENHB 573 and NENHO2 683), and a double mutant construct (748) that combines the NABS 689 and NENHB 573 mutations. In both unstimulated and stimulated macrophages, mutation of the URE (NABS 689) had a modest effect on LTR activity. The importance of the enhancer as a positive regulatory element was more dramatic. Mutations preventing NF-κB binding (NENHB 573, 748, and NENHO2 683) significantly decreased LTR activity in unstimulated macrophages and reduced it to negligible levels in stimulated macrophages.

(iii) Analysis of the promoter element including binding sites for Sp1, TFIID, and UBP-1 (LBP-1) (nucleotides −77 to +1). The promoter region includes the three Sp1 sites, the TATA motif, and some of the reported binding sites for the cellular factor UBP-1 (LBP-1) (8, 20, 21, 40). UBP-1 (LBP-1) interacts with a region of the HIV-1 LTR spanning the TATA box and the untranslated leader region (nucleotides −38 to +27): specifically, a high-affinity site (site I; −16 to +27) and a low-affinity site (site II; −38 to −16). The mutations in Sp1 (SP1ALL 553) and the TATA region (NTATA 2332) abolish binding of Sp1 and TFIID, respectively. In both stimulated and unstimulated macrophages, the role of the promoter region as a dominant regulatory element for mediating basal transcription was clearly shown. Mutation of the Sp1 and TATA binding sites had the most deleterious effect on LTR activity in comparison with the effects on transcription of all the other LTR mutations.

Mutant LTR constructs NTARP 558, NPOLT 560, and NCAP 1581 all contain mutations flanking the TATA region within the low-affinity site II. In stimulated macrophages, the reduction in LTR activity relative to that of the wild type caused by NTARP 558 and NCAP1581 was not as severe as that caused by SP1ALL and NTATA. The NPOLT 560 mutant consistently had no significant effect on LTR activity. In contrast, in unstimulated macrophages the LTR activities of two of the three constructs mutated in the low-affinity site (site II) for UBP-1 (LBP-1) binding (NPOLT 560 and NCAP 1581) were consistently significantly enhanced relative to that of the wild type. Activity of the NTARP 558 construct was more variable but was never less than that of the wild type. This raises the possibility that in unstimulated human macrophages, UBP-1 (LBP-1) binding to site II functions as a repressor of LTR activity and that disruption of certain LBP-1 (UBP-1) binding elements can relieve this repression.

(iv) Analysis of the leader region including binding sites for the transcription factors UBP-1 (LBP-1) and UBP-2 or CTF/NF-1 (nucleotides +1 and +78). The region from +1 to +78 spans the untranslated leader region of the HIV LTR, encompassing the Tat responsive element TAR and sequences that bind the cellular proteins UBP-1 (LBP-1), UBP-2 (+30 to +35 [7]), and CTF/NF1 (+40 to +45 [20]). In the leader region, UBP-1 (LBP-1) recognizes the direct repeat sequence CTCTCTGG. The first such direct repeat (+5 to +12) falls within the high-affinity site (site I [20]) for UBP-1 (LBP-1) binding. Additional leader binding proteins may be required for interaction of UBP-1 (LBP-1) with the second direct repeat

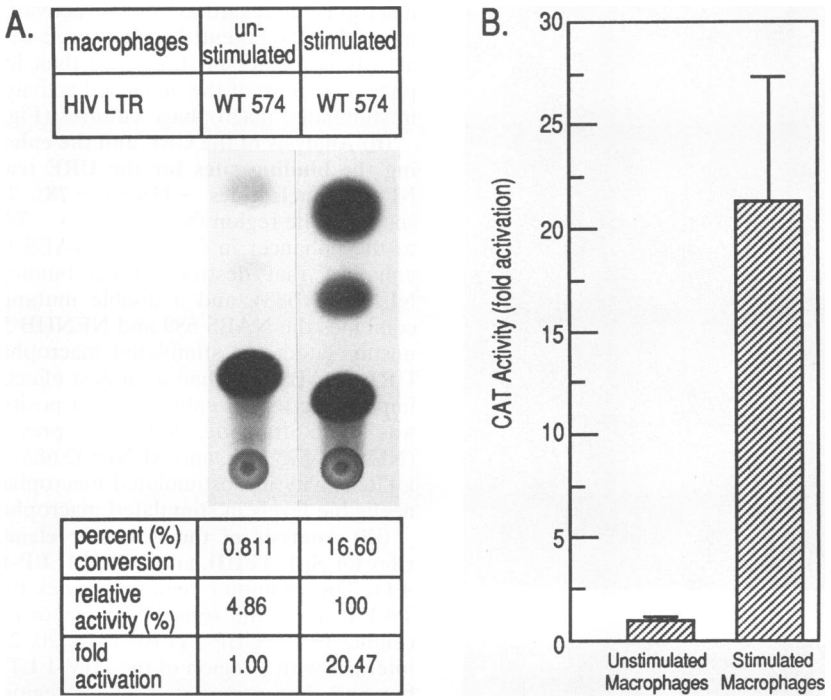

FIG. 3. Effects of macrophage stimulation on LTR activity. Macrophages were either unstimulated or stimulated as described in Materials and Methods and transiently transfected on day 6 of culture with the wild-type LTR (WT574) for 48 h. CAT activity was determined by measuring the amount of acetylated chloramphenicol produced from [^{14}C]chloramphenicol as described in Materials and Methods. Quantitation was performed by liquid scintillation counting. (A) Autoradiographic plate of CAT assays with quantitations shown below the lanes. Both lanes represent transfection with the wild-type LTR (WT574), the variable being the activation state (unstimulated versus stimulated) of the macrophage cultures. One representative experiment is shown. (B) Graphical representation showing the means ± SDs of six independent experiments. LTR activity in stimulated macrophages is expressed as a fold increase relative to LTR activity (adjusted to 1.00) in unstimulated macrophages.

(+37 to +44). The NSENSE 1169 construct contains mutations in both direct repeats but maintains stem base pairing, stem energy, and the primary loop sequence (7). This construct gave significantly reduced LTR activity relative to that of the wild type in both unstimulated and stimulated macrophage cultures. This observation suggests that the site I element for UBP-1 (LBP-1) functions as a positive regulator of HIV transcription in primary human macrophages. The function of additional positive control elements responsive to other leader-binding proteins may also be affected by the mutated direct repeats in the NSENSE mutation.

Thus, it appears that similar sequences function as positive regulatory elements in unstimulated and stimulated macrophages. These elements include the promoter and enhancer elements and to a lesser extent sequences upstream of the enhancer and in the leader region. The differential use of these positive elements that may explain the enhanced wild-type activity seen in stimulated macrophages appears therefore to be quantitative rather than qualitative. Some qualitative differences may also exist in that negative regulatory elements flanking the TATA region were operational in unstimulated macrophages only. Their absence in stimulated macrophages may also contribute to the enhanced wild-type activity seen upon macrophage activation.

Tat-enhanced LTR activity in stimulated and unstimulated macrophages. The viral transactivator protein Tat is essential for HIV replication. We thus performed cotransfection experiments with a *tat* expression vector to evaluate the role of LTR transcriptional control elements for *tat*-enhanced transcription in stimulated and unstimulated macrophages. Importantly, within each state of macrophage activation (unstimulated or stimulated), addition of Tat dramatically enhanced wild-type LTR activity (Fig. 6). In unstimulated macrophages, addition of Tat increased LTR activity up to 20-fold, while in stimulated macrophages an approximately 10-fold increase was consistently observed. The effect of macrophage stimulation on *tat*-enhanced wild-type LTR activity was also considered. Similar to observations made for basal LTR activity in the absence of Tat (Fig. 3), macrophage stimulation in the presence of Tat was associated with an increase in wild-type LTR activity relative to that in unstimulated macrophages. Over a range of six independent experiments (data not shown), this was generally a 5- to 10-fold increase.

To assess the effect of Tat on the control elements previously identified as important for basal LTR activity in unstimulated and stimulated macrophages, gene transfer experiments were duplicated as described above, but Tat was cotransfected into macrophage cultures along with the wild-type or mutant plasmid constructs. The LTR activity of mutant relative to that of the wild type in the presence of Tat was then assessed in both unstimulated (Fig. 7) and stimulated (Fig. 8) macrophages. The activity of each mutant LTR is expressed as a relative percentage of wild-type (WT574) activity.

(i) Analysis of the binding region for the transcription factor USF (nucleotides −177 to −156). In the absence of Tat, mutation of the USF site (NREP 600) significantly reduced LTR activity in both unstimulated and stimulated macrophages. In the presence of Tat, this element no longer appeared essential for optimal LTR activity. Observations from a number of independent experiments showed that in stimulated

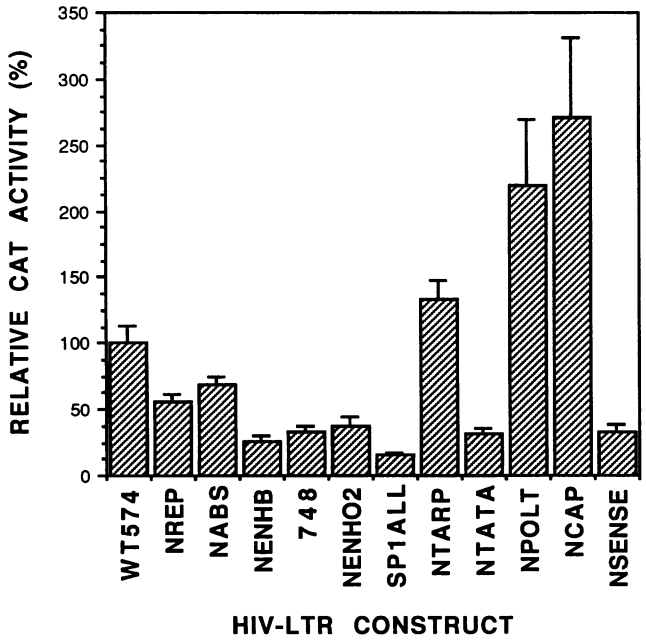

FIG. 4. Transient transfection analysis of the LTR mutants in unstimulated macrophages. Unstimulated macrophages were transfected on day 6 of culture with the wild-type LTR or mutant LTR constructs for 48 h. CAT activity was determined by measuring the amount of acetylated chloramphenicol produced from [^{14}C]chloramphenicol as described in Materials and Methods. Quantitation was performed by liquid scintillation counting. The CAT activity of each mutant LTR is expressed relative to that of the wild-type LTR (WT574); wild-type activity is adjusted to 100%. Shown are the means ± SDs of four independent experiments using macrophages from different donors.

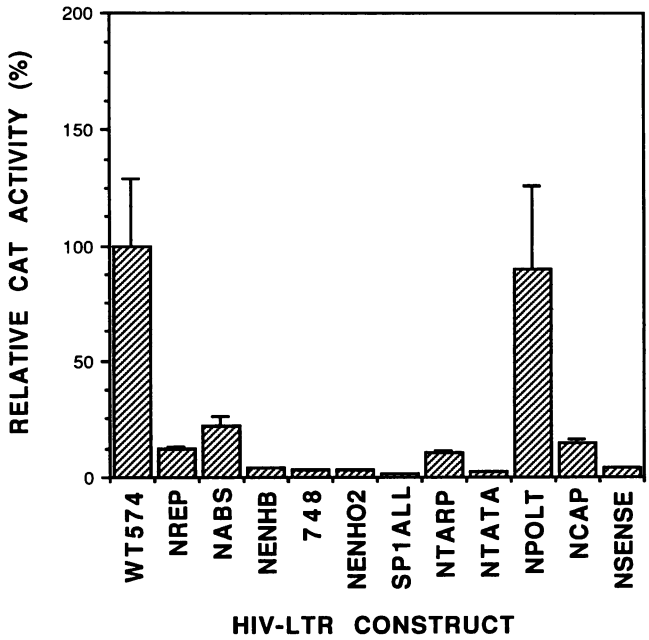

FIG. 5. Transient transfection analysis of the LTR mutants in stimulated macrophages. Macrophages stimulated by initial contact with Con A-activated nonadherent cells were transfected on day 6 of culture with the wild-type LTR or mutant LTR constructs for 48 h. CAT activity was determined by measuring the amount of acetylated chloramphenicol produced from [^{14}C]chloramphenicol as described in Materials and Methods. Quantitation was performed by liquid scintillation counting. The CAT activity of each mutant LTR is expressed relative to that of the wild-type LTR (WT574); wild-type activity is adjusted to 100%. Shown are the means ± SDs of five independent experiments using macrophages from different donors.

macrophages with Tat, there was no significant change in LTR activity, while in unstimulated macrophages with Tat, LTR activity was always equal to or slightly greater than that of the wild type.

(ii) Analysis of the URE and the enhancer element containing the binding sites for the URE transcription factor and NF-κB (nucleotides −156 to −78). In unstimulated macrophages in the presence of Tat, mutation of the URE (NABS 689) caused a modest reduction in LTR activity, while mutation of the enhancer region (NENHB 573, 748, and NENHO2 683) dramatically reduced LTR activity relative to that of the wild type. This trend was similar to that observed in unstimulated macrophage cultures in the absence of Tat. In stimulated macrophages, however, the effect of the URE mutation on Tat-induced LTR activity was less severe than its effect on basal activity, while decreases in the activity of LTR constructs mutated in the enhancer region were insignificant.

(iii) Analysis of the promoter element, including binding sites for Sp1, TFIID, and UBP-1 (LBP-1) (nucleotides −77 to +1). In unstimulated macrophages in the presence of Tat, mutation of Sp1 sites (SP1ALL) and the TATA box (NTATA 2332) resulted in a marked reduction in LTR activity relative to that of the wild type. This suggests that in unstimulated macrophages, the proximal promoter elements Sp1 and the TATA motif contribute a significant role to TAT-induced as well as basal LTR activity. In stimulated macrophages, however, mutation of the Sp1 and TATA elements caused only a slight reduction in TAT-induced LTR activity compared with the deleterious effect of these mutations on basal LTR activity. In both unstimulated and stimulated macrophages, the mutations flanking the TATA box (NTARP 558, NPOLT 560, and NCAP 1581) did not significantly affect Tat-induced LTR activity.

(iv) Analysis of the leader region, including binding sites for the transcription factors UBP-1 (LBP-1) and UBP-2 or CTF/NF-1 (nucleotides +1 and +78). In unstimulated macrophages in the presence of Tat, the mutation within the high-affinity site for UBP-1 (LBP-1) binding (NSENSE 1169) resulted in a reduction in LTR activity comparable to that seen with mutation of the TATA motif. Thus, it appears that the function of this site as an important positive regulator remains in unstimulated macrophages in the presence of Tat. In contrast, in stimulated macrophages, the reduction in Tat-enhanced LTR activity of the NSENSE construct was negligible, suggesting that in the presence of Tat, this element was no longer required for optimal LTR activation in differentiated macrophages.

DISCUSSION

We have established an in vitro model to examine the role of transcriptional control mechanisms in determining the activation status of the HIV LTR in macrophages induced to differentiate by stimulation with activated nonadherent cells. A novel aspect of this system is the reliance on immune macrophage activation and on the local delivery of endogenous cytokines. Thus, this system more closely approximates the physiological mechanisms by which macrophages are activated

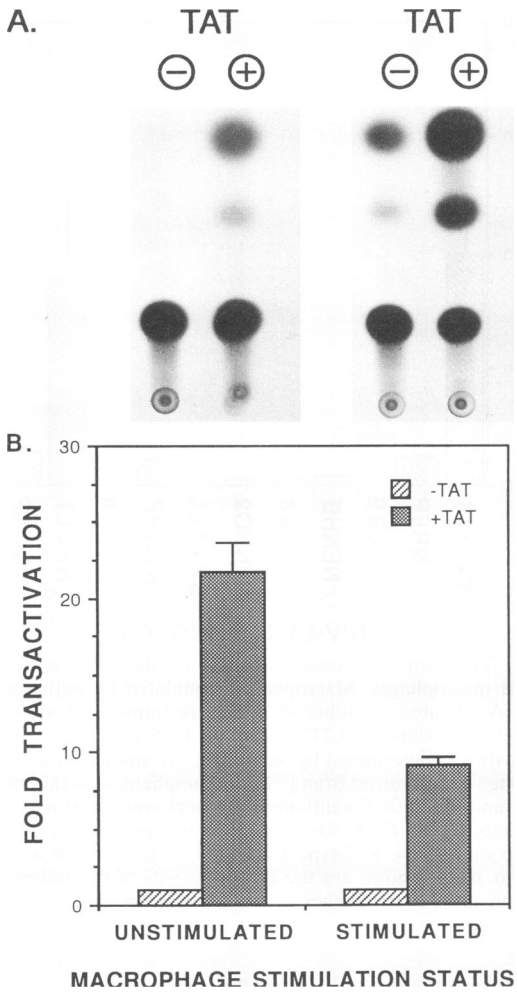

FIG. 6. Effects of Tat on LTR activity in unstimulated and stimulated macrophages. Macrophages were either unstimulated or stimulated as described in Materials and Methods and transiently transfected on day 6 of culture with the wild-type LTR (WT574) for 48 h in the presence or absence of a Tat-containing plasmid introduced by cotransfection. CAT activity was determined by measuring the amount of acetylated chloramphenicol produced from [^{14}C]chloramphenicol as described in Materials and Methods. Quantitation was performed by liquid scintillation counting. (A) Autoradiographic plate of CAT assays performed on unstimulated and stimulated macrophages in the absence (−) or presence (+) of Tat. One representative experiment is shown. (B) Graphical representation showing the means ± SDs of four independent experiments. LTR activity without Tat (basal activity) has been separately adjusted to 1.00 for unstimulated and stimulated macrophages and LTR activity in the presence of Tat expressed as a fold increase relative to basal activity in each case.

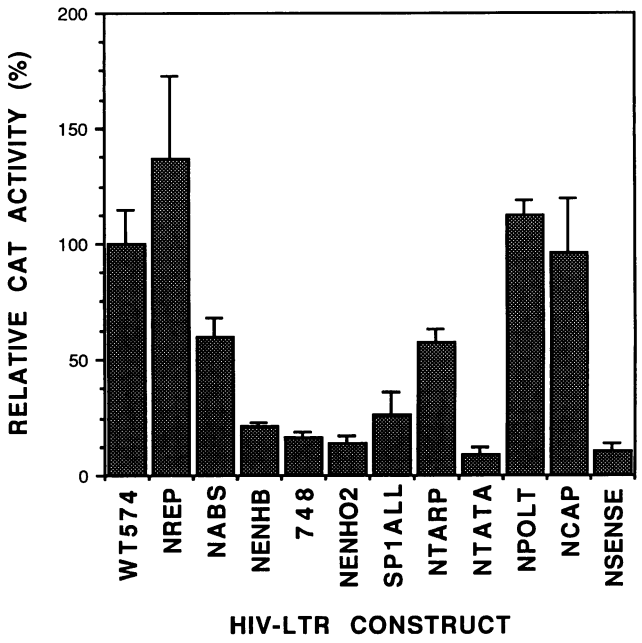

FIG. 7. Transient transfection analysis of the LTR mutants in unstimulated macrophages in the presence of Tat introduced by cotransfection. Unstimulated macrophages were cotransfected on day 6 of culture with the wild-type LTR or mutant LTR constructs and a Tat-containing plasmid for 48 h. CAT activity was determined by measuring the amount of acetylated chloramphenicol produced from [^{14}C]chloramphenicol as described in Materials and Methods. Quantitation was performed by liquid scintillation counting. The CAT activity of each mutant LTR is expressed relative to that of the wild-type LTR (WT574); wild-type activity is adjusted to 100%. Shown are the means ± SDs of four independent experiments using macrophages from different donors.

in vivo than does the use of cell lines and/or the addition of a preselected dose of cytokines as an activation stimulus. Results show that both basal (in the absence of Tat) and Tat-induced wild-type LTR activities are dramatically enhanced upon differentiation of macrophages into multinucleated giant cells. The use of mutant LTR constructs revealed that multiple regulatory elements are able to influence the level of promoter activity. Mutation of not one but several elements debilitated basal LTR activity and *tat*-enhanced activity in undifferentiated macrophages. In differentiated macrophages in the presence of Tat, however, the LTR was more pliable for mutation. Maximal LTR activity was achieved even with the mutation of transcription control sequences that were absolutely required in undifferentiated macrophages.

Regulation of basal transcription. Sequences in the promoter (Sp1 binding sites and the TATA motif) and enhancer (NF-κB binding sequence) regions constituted the most influential positive control elements for basal LTR activity in both stimulated and unstimulated macrophages. The importance of NF-κB, Sp1, and TATA elements in mediating transcriptional activation of the HIV LTR has been shown in T-cell systems (15, 18, 20, 22, 26, 28, 29, 36, 38, 41) and in a monocytic cell line (28). Importantly, the present study identifies these sequences as a vital transcriptional control in primary macrophages.

Sequences upstream of the enhancer (the URE and the USF binding site) functioned as positive regulators of transcription, but their influence was less than that of the enhancer or promoter elements. Using a variety of cell lines, Nakanishi et al. (28) have proposed that the URE participates in either positive or negative regulation of basal-level transcription, depending on the cell type. In the MT-4 and Jurkat T-cell lines, for example, this region functioned negatively, whereas it had a positive effect in the MOLT-4 T-cell line and the monocytic cell line U937. The importance of the USF region also appears to vary with cell type. It has been identified as a negative control element in Jurkat cells in some studies (24, 25), while in other studies a functional USF sequence was necessary for maximal activity of the LTR (41). The importance of these two elements in primary macrophages has not yet been assessed. In

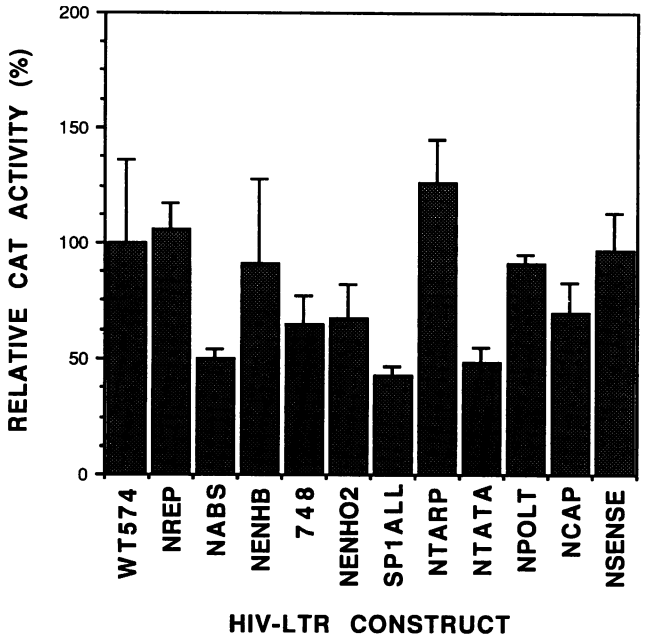

FIG. 8. Transient transfection analysis of the LTR mutants in stimulated macrophages in the presence of Tat introduced by cotransfection. Macrophages stimulated by initial contact with Con A-activated nonadherent cells were cotransfected on day 6 of culture with the wild-type LTR or mutant LTR constructs and a Tat-containing plasmid for 48 h. CAT activity was determined by measuring the amount of acetylated chloramphenicol produced from [^{14}C]chloramphenicol as described in Materials and Methods. Quantitation was performed by liquid scintillation counting. The CAT activity of each mutant LTR is expressed relative to that of the wild-type LTR (WT574); wild-type activity is adjusted to 100%. Shown are the means ± SDs of five independent experiments using macrophages from different donors.

the present study, we find that an intact URE and an intact USF binding site are required for optimal LTR activity.

The high-affinity site (site I) for UBP-1 (LBP-1) in the untranslated leader region was also required for optimal LTR activity. This UBP-1 (LBP-1) binding element has been implicated as a positive control sequence in HeLa cells (20, 21). Kato et al. (21) report that UBP-1 (LBP-1) binding to the high-affinity site enhances transcriptional activity which is dependent on the presence of other factors operating cooperatively or independently. In the present study, the reduced LTR activity seen with the mutant leader construct in primary macrophages suggests the importance of the high-affinity site for UBP-1 (LBP-1), possibly in the context of additional control elements.

The activation state of the HIV LTR may also be influenced by negative control elements. Studies in a number of different cellular systems report the negative influence of the NRE on LTR activity (8, 22, 24, 25, 28, 41). In the primary macrophage system, use of an LTR construct containing upstream NRE sequences caused an up to fivefold decrease in LTR activity relative to that of the wild type in both stimulated and unstimulated macrophages (data not shown). In addition, our study identified a potential negative control mechanism operational in unstimulated macrophages. This mechanism involved sequences flanking the TATA element that fall within the low-affinity recognition site (site II) for UBP-1 (LBP-1). Mutation of these sequences allowed a significant upregulation of LTR activity relative to that of the wild type in the unstimulated macrophages. Kato et al. (21) have reported that in the HeLa cell system, LBP-1 can regulate HIV expression in a dominant negative fashion by interacting with the promoter region and blocking TFIID binding to the TATA element. While at low molar ratios LBP-1 covers only the high-affinity site (site I) and is able to enhance transcription, at high molar ratios it covers both the high- and low-affinity sites and can repress transcription in a dominant manner over upstream positive regulators. In the present study, a similar mechanism may exist; the molar ratio of UBP-1 (LBP-1) to template in unstimulated primary macrophages may be sufficient for repression through allowing occupation of both site I and site II, in the context of a low concentration of positive TATA-binding factors. The mutations flanking the TATA box may enhance LTR activity by rendering the low-affinity site inaccessible to UBP-1 (LBP-1) binding. Thus, in undifferentiated macrophages, UBP-1 (LBP-1) binding to the intact low-affinity site may repress transcription of the HIV LTR, providing a mechanism whereby the provirus favors a transcriptionally latent state. Upon macrophage stimulation and differentiation, this negative control may be relieved, allowing positive control elements to mediate transcriptional upregulation and ultimately reactivation of HIV from the latent state.

The low level of basal transcription from the wild-type HIV LTR in resting relative to differentiated macrophages may be mediated by more than one mechanism. Firstly, although a number of positive regulatory elements are responsive in quiescent cells, their activation potential may be reduced because of a paucity of functionally active cellular transcriptional activators. Secondly, the HIV LTR may be maintained in a relatively inactive state through the influence of negative control elements. These mechanisms may facilitate HIV latency in macrophages in the absence of a distinct immune activation stimulus.

Regulation of *tat*-enhanced transcription. The viral transactivator protein Tat increases HIV expression at the level of transcription initiation and/or elongation, and Tat is essential for replication of HIV in culture (4). As expected, cotransfection of Tat with the viral LTR allowed for enhanced LTR activity in both unstimulated and stimulated macrophages. When *tat* was cotransfected into undifferentiated macrophages along with the mutant LTR constructs, the requirement for multiple intact positive control sequences remained. With the exception of the USF site, mutations in elements identified as essential for optimal basal LTR activity debilitated Tat-mediated LTR activity in undifferentiated macrophages. Promoter and enhancer elements were particularly important for optimal transcriptional activity. Intact upstream promoter and enhancer sequences have been shown to be necessary for Tat-mediated transcriptional activation in certain cell lines (2, 23, 37, 41).

Interestingly, when the mutant LTR constructs were transfected into stimulated macrophages in the presence of *tat*, there was no significant decrease in LTR activity relative to that of the wild-type construct. Thus, sequences previously identified as essential for regulation of LTR activity did not have to be intact to achieve *tat*-mediated enhancement of LTR activity in differentiated macrophages. In Jurkat cells, it has been shown that certain sequences essential for optimal basal transcription are unnecessary for *tat* activation (41). Intact enhancer and promoter sequences were, however, still required for *tat*-mediated transactivation. In contrast, in stimulated primary macrophages, mutation of promoter or enhancer sequences did not debilitate LTR activity. These observations suggest that upon macrophage differentiation, the HIV LTR acquires a degree of flexibility that allows transcriptional

upregulation even if certain cellular factors are limiting or certain transactivation pathways are debilitated. In vivo, stimuli that activate and differentiate macrophages as they traffic through the blood to tissue sites may inadvertently allow reactivation and replication of HIV. A potential for transcriptional control for flexibility within the LTR may allow the virus to exploit these normal mechanisms of immune activation.

In summary, we propose that in quiescent human macrophages, HIV may be held in a latent state because of both the influence of negative control mechanisms and a relative lack of certain cellular transcriptional activators upon which the virus is dependent. Upon macrophage activation, the increased availability of cellular factors may create an environment favoring sufficient transcription from the LTR to allow for production of essential auxiliary viral proteins such as Tat and Rev. In stimulated macrophages, the presence of Tat allows the virus to override a requirement for the cellular environment that is essential for regulation of basal gene expression. With macrophage activation itself augmenting production of *tat*, a positive feedback loop could develop, leading to significant induction of HIV gene expression and virus production. Thus, the acquisition of independence from initial cellular control mechanisms may be a key factor in the progression towards establishing a productive viral infection. Additional cellular factors present in activated macrophages may, however, play an essential role in subsequent Tat- and/or Rev-mediated regulation. Thus, the interaction of both positive and negative macrophage factors with multiple *cis*-acting elements on the HIV LTR appears to represent a novel mechanism that is important both in the establishment and maintenance of latency and in the initial activation steps that lead to establishment of productive HIV infection.

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